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Full paper

Celastrol, an NF-κB inhibitor, ameliorates hypercalciuria and articular cartilage lesions in a mouse model of secondary osteoporosis

Xiaodong Liu, Feng Cai, Yan Zhang, Anli Yang, Liang Liu*

Department of Orthopedics, YangPu Hospital, TongJi University School of Medicine, Shanghai, 200090, People's Republic of China

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ABSTRACT

Notwithstanding compelling contribution of NF-κB to the progression of osteoporosis has been reported, little is known regarding direct inhibition of NF-κB benefiting osteoporosis. In this study, therefore, we evaluated the role of celastrol, an NF-κB inhibitor, in a mouse model of secondary osteoporosis. Animals were divided into three groups as Sham (control), SO (secondary osteoporosis) and SO + CA (secondary osteoporosis treated with celastrol). Significant decreases in body weight and body fat were observed following celastrol treatment in SO group, but leptin levels were much higher. Celastrol also exhibited a significant decrease in urinary calcium excretion. Moreover, other important events were observed after celastrol treatment, covering substantial decrements in serum concentrations of PTH, TRAP-5b, CTX and DPD, improved structure of articular cartilage and cancellous bone (revealed by H&E and safranin-O staining), and significant decline in levels of NF-κB (P65), MMP-1, and MMP-9. These findings demonstrated that celastrol treatment not only improved abnormal lipid metabolism and hypercalciuria in mice subjected to secondary osteoporosis, but also ameliorated articular cartilage lesions. Our results provided evidence of targeted therapy for NF-κB in the clinical treatment of secondary osteoporosis.

1. Introduction

Osteoporosis, a common age-related disease characterized by bone loss and bone microarchitecture deterioration, predominantly affects postmenopausal women (1–3). Primary osteoporosis results from bone loss associated with normal aging, but secondary osteoporosis arises as a result of underlying disorders or medication treatments (4,5). It is generally agreed that hypercalciuria is linked to reduced bone density in osteoporotic patients, especially in postmenopausal women (6,7). A few studies have addressed a detrimental effect of osteoporosis on articular cartilage, suggesting a possible link between osteoporosis and articular cartilage lesions (8,9). A study using a mouse model of periodontitis revealed the relation between lipopolysaccharide-induced bone loss and inflammatory reaction (10). Accumulating knowledge on the pathological mechanisms of osteoporosis also suggested a potential role of inflammatory response in the progression of secondary osteoporosis (11,12).

NF-kappa B (NF-κB), a well-known transcription factor, is an attractive therapeutic target for immune-related diseases due to a pivotal role in regulation of genes that are involved in inflammatory reaction, such as proinflammatory cytokines, adhesion molecules and chemokines (13–15). Li also uncovered the contribution of NF-κB to the progression of osteoporosis induced by type II diabetes (16). To date, many agents have been attempted for the clinical treatment of some inflammatory disease, in view of the anti-inflammatory activity of pharmacological suppression of NF-κB (17–22). Although great advance in pharmacological management of osteoporosis, we still need to explore new agents to lower osteoporotic fractures for better quality of life in the elderly. Celastrol, an NF-κB inhibitor, exerts powerful effects on the treatment of rheumatoid arthritis, chronic inflammation, and neurodegenerative diseases because of its anti-inflammatory activity (23,24), even more, celastrol has been reported as a leptin sensitizer, and a promising agent in the drug therapy for obesity (25), but the knowledge of it's role in

* Corresponding author. Department of Orthopedics, YangPu Hospital, TongJi University School of Medicine, 450 Tengyue Road, Shanghai, 200090, People's Republic of China. Tel.: +86 021 65690520.
E-mail address: dr_liuliang@163.com (L. Liu).
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secondary osteoporosis still remains fragmentary. To elucidate the possible effects of celastrol on secondary osteoporosis, therefore, in the present study, we evaluated lipid metabolism and calcium content following celastrol treatment in a mouse model of secondary osteoporosis, as well as bone metabolism and bone structure.

2. Materials and methods

2.1. Animal experiments and drug administration

Six-week-old male mice (C57BLKS/J), each weighing ranged between 20 and 22 g, were purchased from the Jackson Laboratory (Sacramento, CA, USA). The mice were given free access to food and water and were caged individually under controlled temperature (21–25 °C) and humidity (50–60%) with an artificial light cycle. The animal protocol was approved by the Committee on the Ethics of Animal Experiments of TongJi University. Animals were grouped into three as Sham (control), SO (secondary osteoporosis) and SO + CA (secondary osteoporosis treated with celastrol). Sham group received normal feed; SO group were injected into the intra-articular space of the right knee daily with 5 mg/kg dexamethasone (Sigma, St. Louis, MO, USA) for 8 weeks; SO + CA group were injected daily with a dose of 1 mg/kg celastrol (Sigma, St. Louis, MO, USA).

In addition, for leptin administration, Sham group were divided into three groups: Sham, Sham + Leptin, Sham + LA. Sham group were injected with saline as control; Sham + Leptin group received intraperitoneal injection with 0.1 mg/kg/day leptin for 1 week; Sham + LA group were injected with 20 mg/kg pegylated super-active mouse leptin antagonist (PEG-SMLA) every other day for 4 weeks. So did SO and SO + CA group. The purification, and monopegylation of PEG-SMLA were performed as described previously (26).

2.2. Measurement by dual energy X-ray absorptiometry (DEXA)

The mice were anesthetized with 0.14 mg/kg medetomidine (Domitor, Orion Corporation, Espoo, Finland) to measure body weight, and % body fat was measured by DEXA (Lunar Prodigy, GE Healthcare, Germany) as described before (27).

2.3. Serum calcium and urinary calcium

Serum calcium concentrations were determined in a Technicon-SMAC (Technicon Instruments Corp, Tarrytown, NY). Urinary calcium was measured on 24-h collections by atomic absorption spectrophotometry. An individual was considered hypercalciuric when the 24-h urinary calcium value was ≧0.1 mmol/kg per day on the 20 mmol/day calcium intake.

2.4. Serum biochemical markers of bone and energy metabolism

Anesthetized mice were placed in a euthanasia chamber. Directly after aspiration, the blood was transferred to plain tubes. Serum was centrifuged for 1 min and stored −80 °C until analysis. The serum concentrations of leptin and PTH were assayed at Novartis Pharma Ag with a Luminex 200™ Multiplexing Instrument as described earlier (28). Serum TRAP-5b and ALP-b were measured according to the manufacturer's instructions using an ELISA kit (Linco Research, St. Charles, MO, USA). Serum CTX was detected by an Elecsys β-CrossLaps™ serum assay (Roche Diagnostics) (29). Serum DPD was measured by high-performance liquid chromatography (HPLC) as described previously (30).

2.5. Histomorphological analysis

The cartilage and cancellous bone samples were dissected along the axial plane into pieces of 10 mm × 5 mm × 7 mm with a thin layer of subchondral bone and were fixed in 4% formaldehyde for over 24 h in room temperature. After being decalcified in 10% ethylene diamine tetraacetic acid (EDTA) solution for over 2 weeks, the samples were embedded in paraffin. The specimens were cut into 4 μm thick sections and stained with hematoxylin-eosin (H&E) and safranin-O. We used the structure score according to Mankin histological grading system (31) to evaluate cartilage and cancellous bone degradation. Moreover, cartilage tissues were carefully dissected under a dissecting microscope. The articular surface of each specimen was rinsed with phosphate-buffered saline (PBS). The cartilage surfaces of the femoral condyle and the tibial plateau were examined with a microscope. In addition, chondrocytes were obtained as reported earlier (32), likewise, we also performed safranin-O staining to evaluate the structure of chondrocytes, and used Mankin histological grading system to assess cell score.

2.6. RNA isolation and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cells with Trizol reagent, and further refined by an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Quantitative RT-PCR was performed as reported earlier (33), with some alternations. β-actin was used as the internal control. Primescript RT reagent Kit (Promega, Madison, WI, USA) was used to synthesize cDNA. QRT-PCR reactions were performed using a SYBR Green-containing PCR kit (GenePharma, Shanghai, China). The primers of NF-κB (P65), MMP-1, MMP-9, and β-actin were designed according to Kim et al. (33) and Venugopal et al. (34). The relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ method.

2.7. Western blotting

Western blotting was performed as described previously (33). Briefly, total proteins were extracted from cartilage tissue and chondrocytes using a commercial nuclear extraction kit in accordance with the manufacturer's instructions (Active Motif, Carlsbad, CA). Under denaturing conditions, proteins (35 μg) were separated by 10% SDS-PAGE mini-gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) for 60 min at 100 V. After incubation in blocking buffer (Tris-buffered saline containing 150 mM NaCl, 50 nM Tris, 0.05% Tween-20, pH 7.5) for 1 h at room temperature, the membrane was hybridized in blocking buffer with specific primary antibodies against NF-κB (P65), MMP-1, MMP-9, and β-actin antibody (Santa Cruz, USA) overnight at 4 °C, then incubated with secondary antibodies labeled with horseradish peroxidase (HRP), followed by a detection with chemiluminescence (ECL) reagent (Amersham, Buckinghamshire, UK).

2.8. Statistical analysis

All experiments were carried out at least three times. Data were presented as mean ± standard error of the mean (SEM). Multiple comparisons were performed using the Kruskal–Wallis test with Bonferroni correction, followed by a Mann–Whitney U-test using SPSS 10.0 (SPSS, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of celastrol on lipid metabolism in experimental mice

In view of the known role of NF-κB in controlling feeding behavior (35), we detected body weight and body fat among the three groups. As shown in Fig. 1A and B, SO + CA group exhibited significantly lower body weight and body fat, relative to SO group. Moreover, increase levels of leptin were observed following celastrol treatment (Fig. 1C). Considering the direct influence of leptin on body weight and body fat in experimental mice (Supplementary Fig. 1A and B), and leptin has been reported to act as a crucial regulator of lipid metabolism (36), thus, the results in Fig. 1 suggested that celastrol may alleviate abnormal lipid metabolism, induced by dexamethasone, through regulating leptin levels.

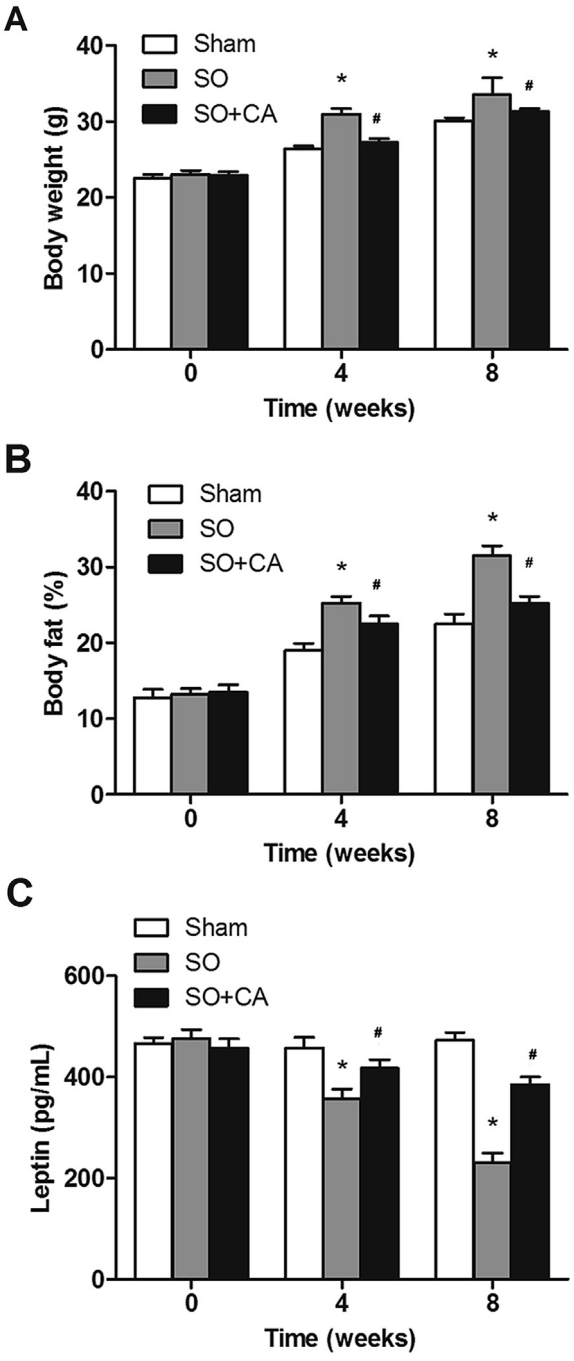

Fig. 1. Effects of celastrol on lipid metabolism in experimental mice (A) body weight (B) body fat (C) leptin level. The data are shown as means ± SEM. *indicates a statistically significant difference between Sham group and SO group ($P < 0.05$). #indicates a statistically significant difference between SO group and SO + CA group ($P < 0.05$).

3.2. Effects of celastrol on serum calcium and urinary calcium in experimental mice

We next examined calcium content between Sham, SO, and SO + CA groups, including serum calcium and urinary calcium. Fig. 2A illustrates a notable decrease in serum calcium content after celastrol treatment, but the significant difference among three groups belonged to the normal fluctuation range (8–12 mg/dL). On the other hand, urinary calcium secretion in celastrol-treated group was significantly lower than secondary osteoporosis group, a statistically significant difference (Fig. 2B). These findings indicated that hypercalciuria was improved in mice with secondary osteoporosis after celastrol treatment.

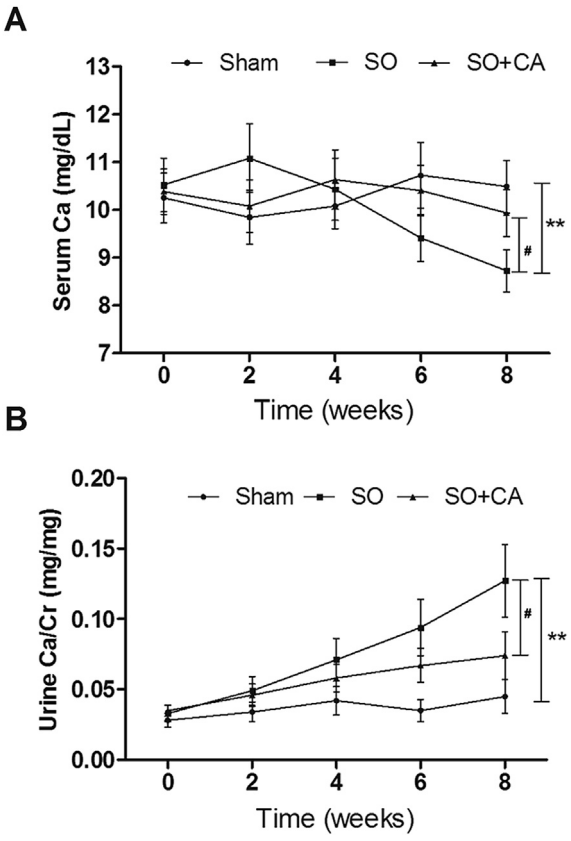

Fig. 2. Effects of celastrol on serum calcium and urinary calcium excretion in experimental mice (A) serum calcium: a statistically significant decrease in serum calcium content was observed in SO group compared with Sham group (**$P < 0.01$), and SO + CA group exhibited significantly higher serum calcium content, relative to SO group (#$P < 0.05$). (B) Urine calcium: SO group exhibited higher urinary calcium excretion compared with Sham group (**$P < 0.01$). SO + CA group exhibited significantly lower urinary calcium excretion, relative to SO group (#$P < 0.05$). The data are expressed as means ± SEM.

3.3. Effects of celastrol on serum markers of bone turnover in experimental mice

We further investigated several serum markers of bone metabolism among three groups. Significant decreases in serum concentrations of PTH and TRAP-5b were seen following celastrol treatment, but ALP-b serum concentrations did not differ significantly between SO group and SO + CA group (Fig. 3A–C). More interestingly, collagen C-terminal telopeptide (CTX) and deoxypyridinoline (DPD), the serum markers of cartilage metabolism, were also significantly reduced with celastrol treatment in SO group (Fig. 3D and E). Above results suggested that celastrol treatment led to striking improvement in bone metabolism, as well as in cartilage metabolism.

3.4. Effects of celastrol on articular cartilage in experimental mice

Based on the known effect of celastrol on improving bone metabolism, H&E and safranin-O staining were performed to assess bone structure in mice with secondary osteoporosis. As shown in Fig. 4A, safranin-O staining of articular cartilage was reduced in secondary osteoporosis group, while celastrol treatment enhanced the staining. Likewise, H&E staining revealed the degradation of cancellous bone in proximal tibia metaphysis in mice with secondary osteoporosis, however, celastrol led to obvious amelioration of cancellous bone degradation. Moreover, the bone structure scores exhibited a parallel trend as the staining results among three groups. SO + CA group got a much lower structure score of articular cartilage than SO group, as well as of cancellous bone (Fig. 4B). Furthermore, safranin-O staining revealed chondrocytes hypertrophy in SO group, but celastrol displayed compelling amelioration of cellular abnormality and irregularity, which was further confirmed through cell score (Fig. 5). Our results indicated that celastrol treatment led to compelling amelioration of articular cartilage lesions in mice with secondary osteoporosis.

3.5. Effects of celastrol on proteins involved in cartilage metabolism in experimental mice

To further confirm the effects of celastrol on articular cartilage in experimental mice subjected to secondary osteoporosis, levels of

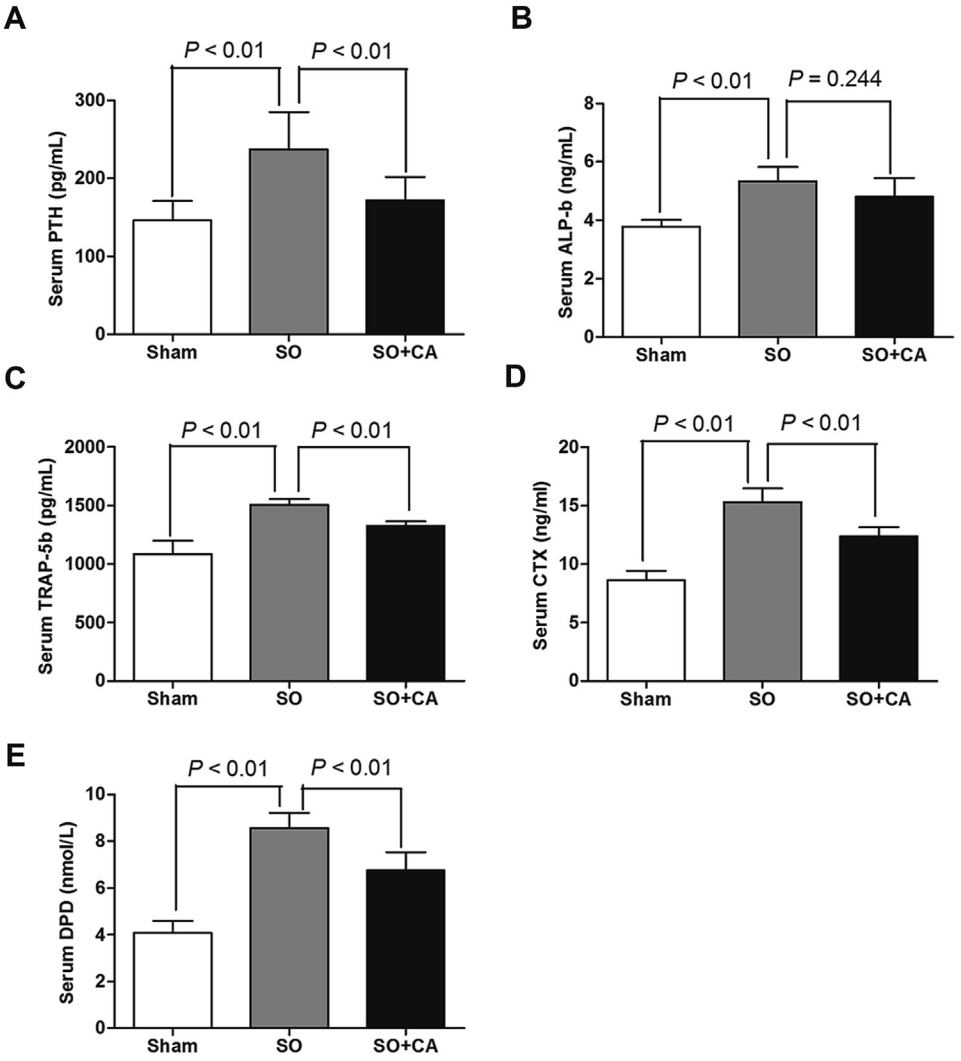

Fig. 3. Concentrations of serum PTH (A), ALP-b (B), TRAP-5b (C), CTX (D) and DPD (E) in experimental mice. Data are shown as the means ± SEM.

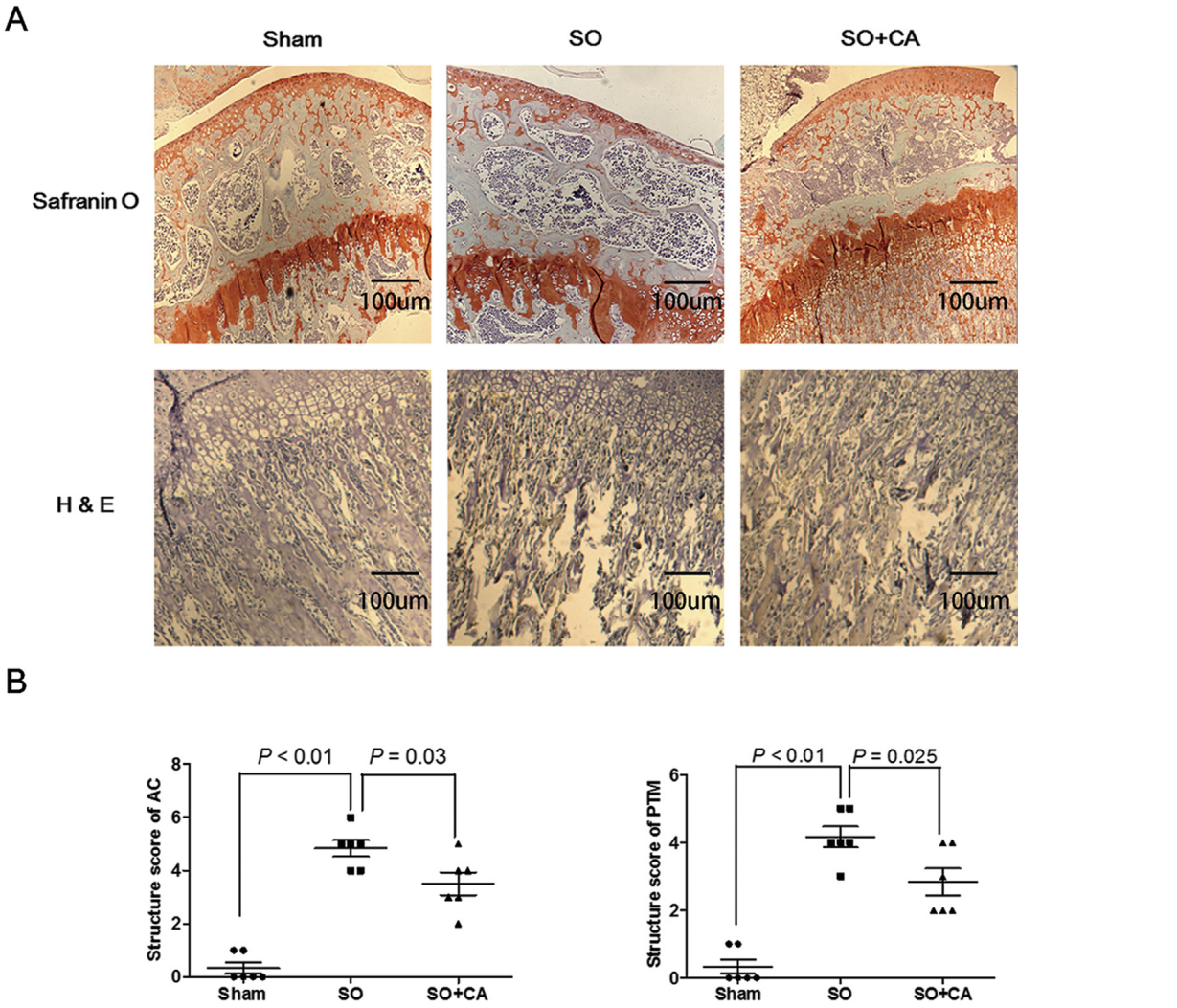

Fig. 4. Histological evaluation of bone structure after treatment with celastrol in experimental mice. (A) Safranin-O staining of articular cartilage, and H&E staining of cancellous bone in proximal tibia metaphysis per group. The articular cartilage staining was reduced in secondary osteoporosis group. (B) Structure score of articular cartilage (AC) and cancellous bone in proximal tibia metaphysis (PTM). Bone structure was graded using Mankin scoring system. The data are expressed as means ± SEM. SO group got significantly higher structure scores of AC and PTM than Sham group ($P < 0.01$). SO + CA group got much lower structure scores of AC and PTM than SO group ($P = 0.03$, $P = 0.025$).

proteins involved in cartilage metabolism, including NF-κB (P65), MMP-1, and MMP-9, were investigated among three groups. Obviously, NF-κB (P65), MMP-1, and MMP-9 levels in SO group were much higher than Sham group, but significant decline in levels of these proteins were observed following celastrol treatment (Fig. 6A and B).

4. Discussion

Current knowledge on the pathogenesis of osteoporosis suggested a potential role of inflammatory response in the progression of secondary osteoporosis. NF-κB, a key factor in inflammatory system, is responsible for the expression of genes that are involved in inflammatory reaction. Till now, a great deal of evidences have revealed the beneficial effect of direct inhibition of NF-κB on inflammatory disease (37,38), thereby, in this study, we assessed body weight, body fat, serum and urinary calcium, serum markers of bone metabolism, and morphological changes in bone structure in a mouse model of secondary osteoporosis induced by dexamethasone, after celastrol treatment. We observed marked decreases in body weight and body fat in mice with secondary osteoporosis after celastrol treatment, and also a significant increase in leptin level. The possibility of significant weight loss is reduced food consumption after celastrol treatment (33). Leptin is presumably to activate hypothalamic NF-κB and modulate the transcription of leptin-stimulated POMC, thus, regulated lipid metabolism (39). Decreased body weight in illness-and leptin-induced anorexia has been reported to be mediated through NF-κB activation in hypothalamic pro-opiomelanocortin (POMC) neurons (40). Additionally, in this study, we confirmed the direct effect of leptin on body weight and body fat in experimental mice after celastrol administration, strongly indicating that celastrol exerted clear effects on regulating abnormal lipid metabolism in mice subjected to secondary osteoporosis, through affecting leptin expression.

Urinary excretion of bone-seeking labels, which is more practical and precise than biomarkers, provides a novel insight

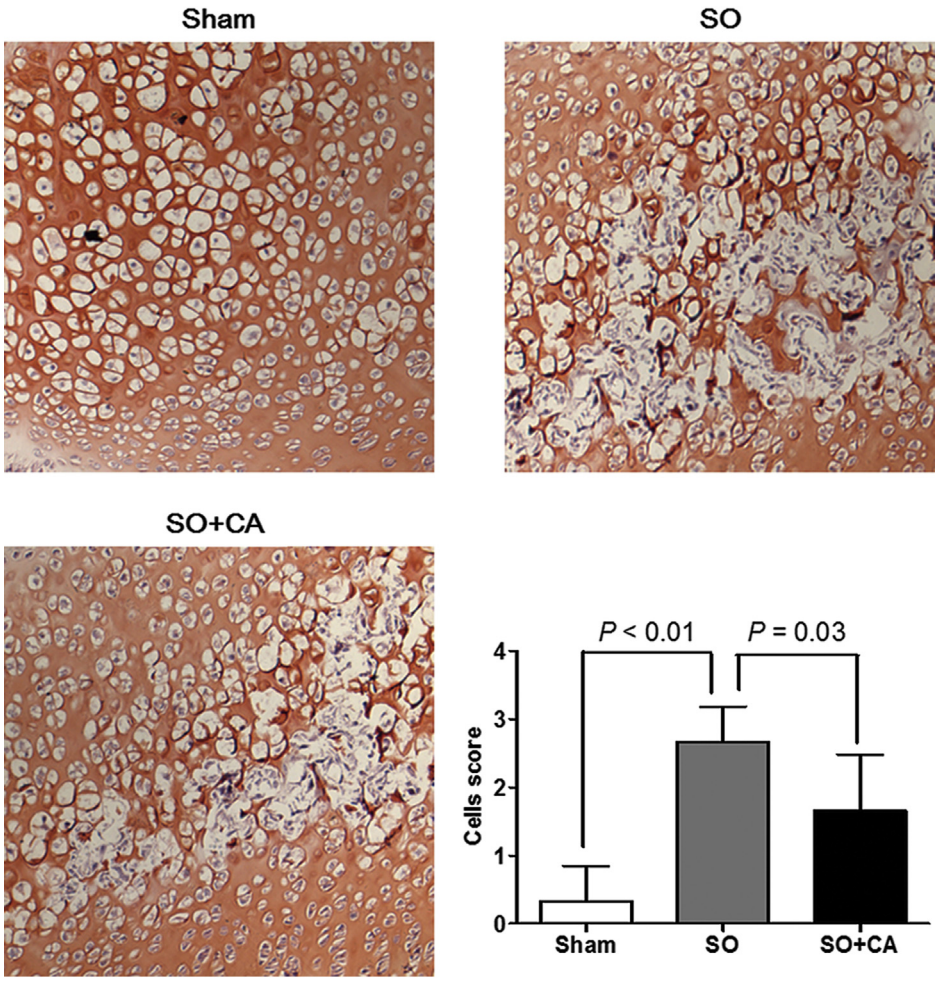

Fig. 5. Effects of celastrol on chondrocytes in experimental mice, and cell score was performed to assess chondrocytes through Mankin scoring system. Data are expressed as the means ± SEM.

for evaluating bone turnover (41). Ca isotopes are preferable for assessing bone turnover, considering that they are more effective in labeling the skeleton (42), as well as are easily reabsorbed into newly forming bone (43). Thus, urinary calcium excretion is responsive to the changes in bone turnover. As elevated urinary calcium frequently occurs in osteoporotic subjects, especially in postmenopausal women with osteoporosis (7), in this study, we further found that celastrol treatment in experimental mice with secondary osteoporosis led to significant improvement in hypercalciuria, and also in hypercalcemia, but notable changes in serum calcium between SO group and SO + CA group belonged to the normal fluctuation range, indicating, hypercalciuria, a promising indicator of secondary osteoporosis for clinical therapy, is dramatically ameliorated after celastrol treatment.

On the other hand, we also found significant decline in serum concentrations of PTH, TRAP-5b, CTX and DPD, the commonly used serum bone markers, in experimental mice with secondary osteoporosis after celastrol treatment, indicating the ameliorative effect of celastrol on abnormal bone metabolism in secondary osteoporosis. Osteoblasts are in charge of bone formation and osteoclasts for its resorption, and the balance of bone metabolism is sustained through the coordination of these two. Changes in serum bone markers not only predict the response in bone metabolism (44), but also can be detected earlier than alterations in bone mineral density (BMD) (45). Many studies have addressed the relevance between serum markers of bone metabolism and osteoporosis. In the U.S., Australia, even Europe, they have started intermittent PTH injection to excite bone formation (46). Actually, the use of PTH in therapy for osteoporosis has already been established (47). In our study, H&E and safranin-O staining also revealed compelling amelioration of articular cartilage lesions and degradation of cancellous bone in mice with secondary osteoporosis after celastrol treatment. It was previously reported that microstructural alterations of articular cartilage were closely related to the progression of osteoporosis, and also osteoarthritis (48). Furthermore, celastrol exhibited chondroprotective properties in a mouse model of secondary osteoporosis. Following a recent study, decreased MMP expression is associated with NF-κB signaling pathway in human osteoarthritic chondrocytes (49). Our study demonstrated that the expression of NF-κB (P65), MMP-1, and MMP-9 were all significantly reduced with celastrol treatment in experimental mice with secondary osteoporosis.

In summary, celastrol not simply exhibited dramatic amelioration of disordered lipid metabolism and hypercalciuria in a mouse model of secondary osteoporosis induced by dexamethasone, but also mitigates articular cartilage lesions, both in cartilage tissues and chondrocytes. Our results strongly implicate NF-κB as a novel therapeutic target in the future treatment of secondary osteoporosis.

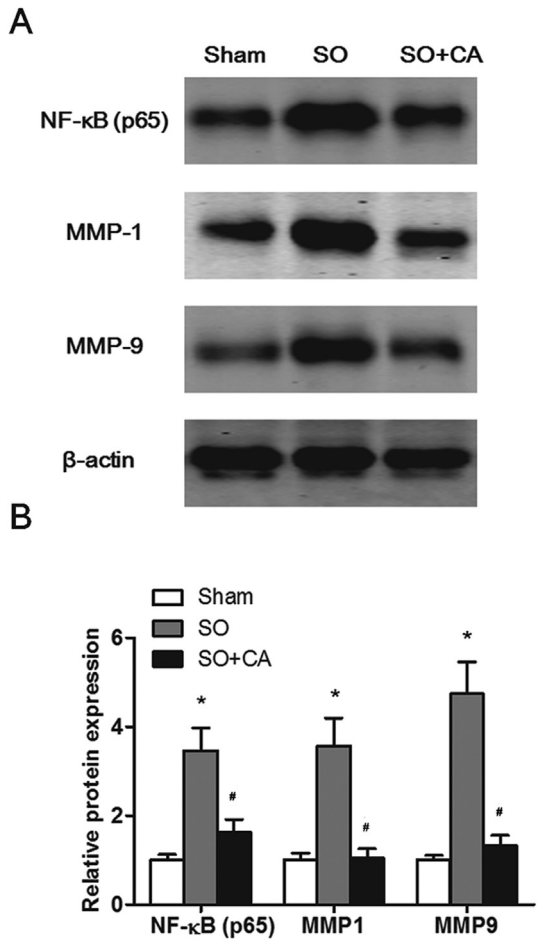

Fig. 6. Effects of celastrol on the expression of NF-κB (P65), MMP-1, and MMP-9 in experimental mice (A) Protein levels of NF-κB (P65), MMP-1, and MMP-9 were determined by western blot, and β-actin served as internal control. (B) mRNA levels of NF-κB (P65), MMP-1, and MMP-9 were determined by qRT-PCR. GAPDH was used as internal control. The data are shown as means ± SEM. *indicates a statistically significant difference between Sham group and SO group ($P < 0.05$). #indicates a statistically significant difference between SO group and SO + CA group ($P < 0.05$).

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2016.02.001.

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